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GCR1, a transcriptional activator in *Saccharomyces cerevisiae*, complexes with RAP1 and can function without its DNA binding domain

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In *Saccharomyces cerevisiae*, efficient expression of glycolytic and translational component genes requires two DNA binding proteins, RAP1 (which binds to UAS_{RPG}) and GCR1 (which binds to the CT box). We generated deletions in *GCR1* to test the validity of several different models for GCR1 function. We report here that the C-terminal half of GCR1, which includes the domain required for DNA binding to the CT box *in vitro*, can be removed without affecting GCR1-dependent transcription of either the glycolytic gene *ADH1* or the translational component genes *TEF1* and *TEF2*. We have also identified an activation domain within a segment of the GCR1 protein (the N-terminal third) that is essential for *in vivo* function. RAP1 and GCR1 can be co-immunoprecipitated from whole cell extracts, suggesting that they form a complex *in vivo*. The data are most consistent with a model in which GCR1 is attracted to DNA through contact with RAP1.

Key words: activation domain/*ADH1*/glycolytic genes/heteromer/transcription initiation

Introduction

Efficient transcription of glycolytic genes in *Saccharomyces cerevisiae* requires the GCR1 protein (glycolysis regulator) (Clifton *et al.*, 1978). The level of most glycolytic enzymes is $<10\%$ of normal in the *gcrl-1* strain (Clifton and Fraenkel, 1981). GCR1 appears to act by increasing transcription of its target genes (Baker, 1986; Holland *et al.*, 1987; Santangelo and Tornow, 1990), but is probably not a general transcription factor. For example, the *gcrl-1* mutation does not affect expression of isocitrate dehydrogenase or glucose-6-phosphate dehydrogenase (Clifton and Fraenkel, 1981). *GCR1* has been cloned; its product has a predicted molecular weight of 94 kDa (Baker, 1986; Holland *et al.*, 1987). GCR1 binds *in vitro* to a CTTCC sequence motif (hereafter referred to as the CT box) found in some glycolytic promoters (Baker, 1991), and recent evidence suggests that it contacts the CT box in *TP1 in vivo* (Huie *et al.*, 1992). Based on this observation, it has been proposed that GCR1 binds to DNA and activates transcription of target genes, either alone (Huie *et al.*, 1992) or as part of a complex with GCR2 [in which GCR1 provides the DNA binding domain and GCR2 provides the activation domain (Uemura and Jigami, 1992)]. Both of these models predict that the DNA binding domain of GCR1 is an essential part of the protein and that loss of that domain should abolish GCR1-dependent transcription.

Efficient expression of glycolytic genes (e.g. *ADH1, PYK1, TPI1, ENO1* and *TDH3*) also requires UAS_{RPG} (upstream activating sequence in ribosomal protein genes), the binding site in DNA for the multifunctional protein RAP1 [repressor/activator protein (Chambers *et al.*, 1988; Gerster and Roeder, 1988; Machida *et al.*, 1988; Tornow and Santangelo, 1990; Bitter *et al.*, 1991)]. UAS_{RPG} is found in the promoters of many genes, including, in addition to glycolytic genes (Tornow and Santangelo, 1990), most of the ribosomal protein genes and the genes encoding elongation factor 1α [*TEF1* and *TEF2* (Huet *et al.*, 1985; Huet and Sentenac, 1987)]. We have found that just as many GCR1-dependent genes are UAS_{RPG}-dependent, several non-glycolytic UAS_{RPG}-dependent genes (*CRY1, TEF1, TEF2*) are also GCR1-dependent (Santangelo and Tornow, 1990). In addition, we have reported that GCR1 can act through a single, isolated UAS_{RPG} element in the absence of a CT box (Santangelo and Tornow, 1990). Based on these data, we thought it possible that RAP1 and GCR1 activate transcription interdependently, perhaps by forming a heteromeric complex. According to this hypothesis, the DNA binding domain of GCR1 may not play as important a role.

To distinguish among these models for GCR1 function, we generated two C-terminal truncation mutants of GCR1, both of which lack the entire specific DNA binding domain (Huie *et al.*, 1992). We found that both of these truncated GCR1 proteins were capable of complementing the growth defect in *gcr1*$^-$ cells and restoring transcription of *ADH1, TEF1* and *TEF2*. We also report here that RAP1 and GCR1 can be co-immunoprecipitated from whole cell extracts. Finally, we demonstrate that GCR1 contains an activation domain and that retention of the region containing the activation domain is essential for GCR1 function *in vivo*. The data are best explained by a model in which GCR1 provides an activation domain to a heteromeric complex containing the UAS_{RPG}-binding protein RAP1.

Results

CT boxes are insufficient for GCR1-dependent activation of ADH1 transcription

If GCR1 activates transcription (either alone or in a complex with GCR2) through binding at the CT box, then it should be possible for the CT box to act as a UAS element. Bitter *et al.* (1991) have demonstrated that the CT box does not by itself activate transcription when inserted upstream from a *lacZ* reporter gene. We therefore wished to determine if, in the *ADH1* promoter, CT boxes could mediate GCR1-dependent transcriptional activation in the absence of RAP1 binding sites. We have shown that *GCR1* disruption

Table I. Effect of abolishing RAP1 binding on transcriptional activation by GCR1

	β-galactosidase units/mg protein[a]	
	GCR1$^+$	gcr1$^-$
pPC0(−615)UAS$_{RPG}$[b]	2082 ± 193[c]	615 ± 106
pPC0(−615)UAS$_{RPG}$*	546 ± 98	481 ± 24
pPC0	<1	ND[d]

[a]LAC4 expression (β-galactosidase activity) was measured in units/mg protein as described in Santangelo *et al.* (1988).
[b]Oligonucleotides containing either the wild type RAP1 binding site (UAS$_{RPG}$) and *ADH1* or a mutant RAP1 binding site (UAS$_{RPG}$*) were inserted just upstream from the *ADH1* promoter sequences in pPC0(−615). The double-stranded UAS$_{RPG}$* and UAS$_{RPG}$ oligonucleotides differ at a single position; RAP1 does not bind to UAS$_{RPG}$* (Nieuwint *et al.*, 1989).
[c]Standard error of the mean of three independent determinations.
[d]ND, not determined.

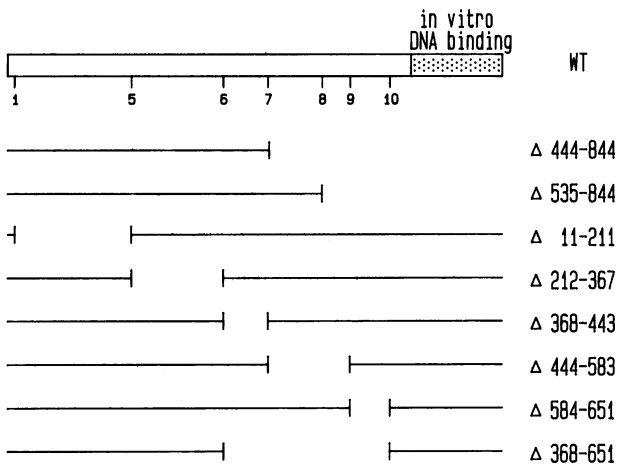

Fig. 1. Location of deletion mutations. Also shown is the location of the *in vitro* DNA binding domain [stippled box (Huie *et al.*, 1992)]. The mutants are named to reflect the amino acids that have been removed. The relevant restriction sites used for the deletion mutagenesis are indicated as follows: 1, *Xmn*I; 5, *Nco*I; 6, *Pvu*I; 7, *Ava*I; 8, *Pvu*II; 9, *Tth*111I; 10, *Ssp*I.

severely impairs the *ADH1* promoter, which is strongly dependent on a single UAS$_{RPG}$ element (Santangelo and Tornow, 1990; Tornow and Santangelo, 1990). To test the possibility that other sequences in the *ADH1* promoter, including several potential GCR1 binding sites (Huie *et al.*, 1992), could independently account for part of GCR1's involvement in *ADH1* transcription, a point mutation that eliminates RAP1 binding (Nieuwint *et al.*, 1989) was introduced into the UAS$_{RPG}$ element in the *ADH1* promoter. The wild type promoter activated high levels of reporter gene expression; most of this activation required GCR1 (Table I). The point mutation reduced the level of reporter enzyme, as expected; the residual level of β-galactosidase was not affected by the presence or absence of GCR1 (Table I). The RAP1- and GCR1-independent *ADH1* transcription detected in Table I is mediated by a 33 bp pyrimidine-rich sequence (−336 to −369 with respect to the *ADH1* mRNA start site) that does not contain a CT box (J.Tornow and G.M.Santangelo, unpublished data). These results suggest that GCR1 function depends on the presence of a UAS$_{RPG}$ element.

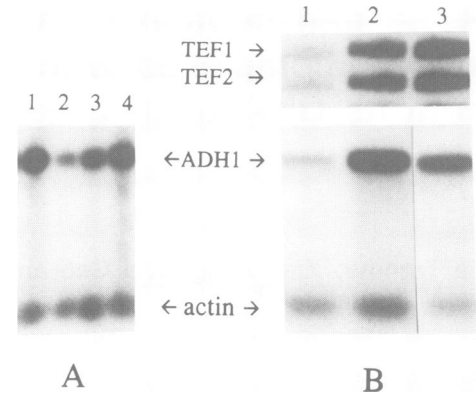

Fig. 2. Stimulation of transcription by truncated GCR1 proteins. Equal amounts of total RNA (from *gcr1*$^-$ transformants harvested during early logarithmic phase) were subjected to S1 nuclease analysis. **A.** S1-resistant products were separated in alkaline 2% agarose gels (*ADH1* and actin; panel A and B) or 5% polyacrylamide, 7 M urea gel (*TEF1* and *TEF2*; panel **B**). Lanes, panel A, *gcr1*$^-$ transformants containing: 1, wild type *GCR1* gene; 2, no *GCR1* gene; 3, the truncation mutant Δ444−844; 4, the truncation mutant Δ535−844. Lanes, panel B, *gcr1*$^-$ transformants containing: 1, no *GCR1* gene; 2, wild type *GCR1* gene; 3, the truncation mutant Δ444−844.

Truncated GCR1 proteins that lack the DNA binding domain activate transcription

A possible explanation for the failure of an isolated CT box to act as a UAS (Bitter *et al.*, 1991) is that GCR1 binds to the CT box only after RAP1 binds to a nearby UAS$_{RPG}$ element. This model, which also explains the dependence of GCR1 function on the presence of a RAP1 binding site (Table I), suggests that RAP1 somehow changes the conformation of the promoter DNA, making it accessible to GCR1. The DNA binding domain of GCR1 should then be essential to GCR1-dependent transcription. To test this, we constructed two mutant GCR1 genes, Δ444−844 and Δ535−844, which encode proteins truncated at amino acids 444 and 535, respectively (Figure 1). Both of these proteins lack the C-terminal DNA binding domain, which is located between amino acids 690 and 844 (Huie *et al.*, 1992). A fusion protein containing the 690−844 segment of GCR1 binds to the CT box *in vitro*, whereas GCR1 proteins truncated at residues 431, 594 and 690 do not (Huie *et al.*, 1992).

Contrary to predictions of the conventional models for GCR1 function (Huie *et al.*, 1992; Uemura and Jigami, 1992), GCR1-dependent *ADH1* transcription was at least partially restored by the Δ444−844 and Δ535−844 proteins (Figure 2A, lanes 3 and 4). In fact, the Δ535−844 protein generated wild type levels of *ADH1* mRNA (Figure 2A, lane 1). We also measured the levels of *TEF1* and *TEF2* transcripts in Δ444−844 transformants. We found that transcriptional activation of *TEF1* and *TEF2* by GCR1 appeared to be unaffected or even slightly improved by removal of the GCR1 DNA binding domain (Figure 2B, lane 3). The ability of the genes encoding truncated GCR1 proteins to restore transcription of GCR1-dependent genes was matched by their capacity to complement the slow growth phenotype of *gcr1*$^-$ cells (Clifton and Fraenkel, 1981). Δ444−844 transformants generated colonies (Figure 3c) that were intermediate in size between wild type and *gcr1*$^-$ cells (Figure 3a and b, respectively), while Δ535−844 transformants generated colonies that were wild

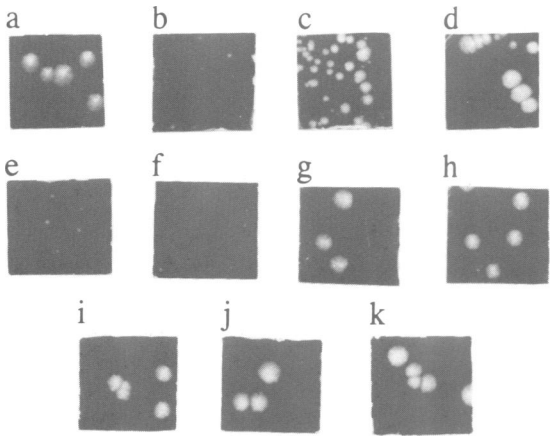

Fig. 3. Complementation analysis of *GCR1* deletion mutants. *gcr1*$^-$ cells transformed with derivatives of the multicopy plasmid YEp13 were plated onto YNBD agar. Colonies were photographed after 10 days of growth at 30°C. **a**, *gcr1*$^-$ cells transformed with wild type *GCR1*: **b**, untransformed *gcr1*$^-$ cells; c–i, *gcr1*$^-$ cells transformed with: **c**, Δ444–844; **d**, Δ535–844; **e**, Δ11–211; **f**, Δ212–367; **g**, Δ368–443; **h**, Δ444–583; **i**, Δ584–651; **j**, Δ368–651; **k**, myc-tagged wild type *GCR1*.

type in size (Figure 3d). These results indicate that the growth of the cells is roughly correlated with the capacity of the GCR1 protein to stimulate transcription, and that it does so even after its specific DNA binding domain has been removed.

GCR1 and RAP1 form a complex

The above results suggested that specific DNA binding by GCR1 is not the primary means by which it is targeted to promoter regions. A possibility raised by our previous work on GCR1–RAP1 interdependence is that GCR1 is part of a UAS_{RPG}-bound heteromeric complex. Previous attempts to detect GCR1–RAP1 complexes by looking for supershifts (Santangelo and Tornow, 1990) or other comparisons of GCR1$^+$ and gcr1$^-$ cell extracts by band retardation analysis (Scott *et al.*, 1990) were unsuccessful. We therefore assayed for GCR1–RAP1 complexes by co-immunoprecipitation. To reduce the possibility of antibody interference with complex formation, we modified the *GCR1* gene by inserting two copies of a 10 amino acid myc epitope. We then used strains generated by Kim Arndt (Cold Spring Harbor Laboratory) that expressed the myc-tagged GCR1 protein and either unmodified RAP1 or RAP1 protein that was tagged with two copies of the hemagglutinin (HA) epitope. A monoclonal antibody directed against the HA epitope (also generously provided by Kim Arndt) was used to immunoprecipitate the HA-tagged RAP1 protein from freshly prepared, ^{35}S-labeled whole cell extracts. The immunoprecipitated samples were analyzed by SDS–PAGE for the presence of RAP1 (Figure 4A, untagged RAP1, lane 1; HA-tagged RAP1, lane 2). Co-immunoprecipitation of GCR1–myc_2 by the anti-HA antibody was detected by Western blot analysis using a monoclonal antibody directed against the myc epitope. The myc-tagged GCR1 protein was co-immunoprecipitated by the HA antibody from cell extracts containing HA-tagged RAP1, but not from cells containing untagged RAP1 (Figure 4B, lanes 2 and 1, respectively).

Since RAP1 and GCR1 are both DNA binding proteins, it was possible that the two proteins were co-immunoprecipitated not because they were complexed with each other, but because they were tethered together as a consequence of binding to DNA in the whole cell extract. To address this concern we repeated the immunoprecipitation in the presence of ethidium bromide (EtBr), which inhibits DNA–protein interactions (Lai and Herr, 1992). To ensure that EtBr inhibited DNA binding by RAP1, we preincubated whole cell extracts with radiolabeled oligonucleotide (containing two copies of UAS_{RPG}) and then added increasing amounts of EtBr (Figure 4C). DNA binding by RAP1 was completely inhibited by the presence of 80 μg/ml EtBr (Figure 4C, lane 4). However, GCR1 was co-immunoprecipitated by the HA antibody despite the addition of up to 400 μg/ml EtBr (Figure 4B, lanes 3, 4 and 5). Although intercalating agents like EtBr can disrupt protein–protein interactions, GCR1–RAP1 complex formation is affected only slightly by EtBr and only at the highest concentration tested (Figure 4C, lane 5). These results suggest that RAP1 and GCR1 form a heteromeric complex.

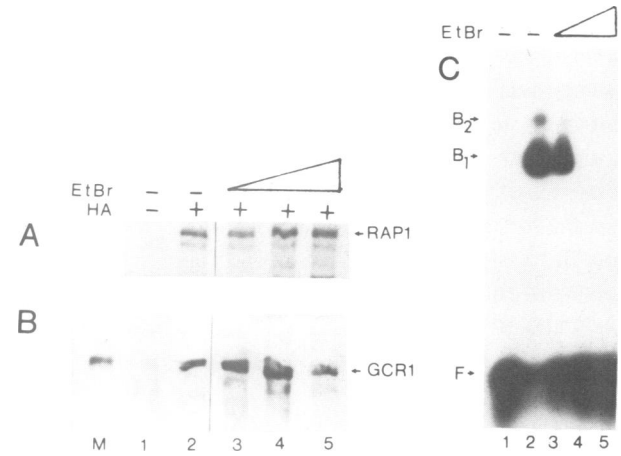

Fig. 4. RAP1 and GCR1 form a complex *in vitro* in a DNA-independent manner. **A.** Whole cell extracts of ^{35}S-labeled yeast cells containing myc-tagged GCR1 and either untagged RAP1 (strain CY1658, lane 1) or HA-tagged RAP1 (strain CY1662, lanes 2–5) were pretreated, where indicated, with EtBr at a final concentration of 16 μg/ml (lane 3), 80 μg/ml (lane 4) or 400 μg/ml (lane 5) and immunoprecipitated by using anti-HA antibody. The immunoprecipitates were analyzed by SDS–PAGE. The position of RAP1 is indicated. **B.** Unlabeled whole cell extracts of strain CY1658 (untagged RAP1) and strain CY1662 (HA-tagged RAP1) were immunoprecipitated by using anti-HA antibody. The immunoprecipitates were analyzed by Western blot analysis with the anti-myc antibody. The position of GCR1 is indicated. Lanes: M, whole cell extract (not immunoprecipitated) of CY1662 to show position of GCR1; 1–5, same as in panel A. **C.** Band retardation analysis was used to demonstrate that RAP1 binding to DNA is eliminated at EtBr concentrations that do not interfere with RAP1–GCR1 complex formation. Radiolabeled UAS_{RPG} oligonucleotide was preincubated with whole cell extract of CY1662 cells to allow RAP1–DNA complex formation. EtBr was then added, where indicated. Lanes: 1, no extract added; 2, no EtBr added; 3–5, EtBr added to a final concentration of 16 μg/ml (lane 3), 80 μg/ml (lane 4) and 400 μg/ml (lane 5). F, free DNA; B_1, oligonucleotide bound by one RAP1 molecule; B_2, oligonucleotide bound by two RAP1 molecules.

The N-terminal third of GCR1 contains an activation domain

To test for the existence of an activation domain in GCR1, we constructed fusion proteins containing the DNA binding

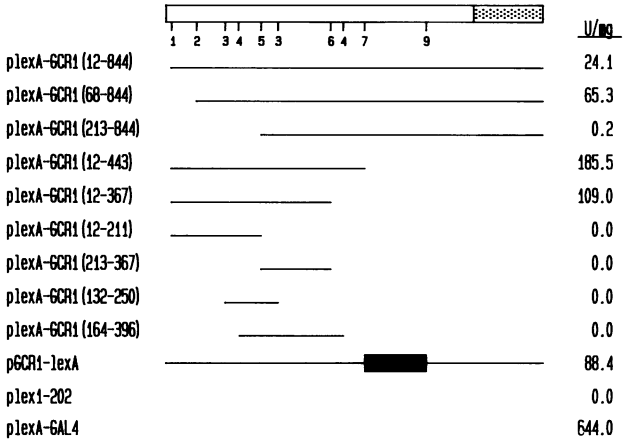

Fig. 5. Identification of an activation domain in GCR1. All of the fusion genes except one were constructed by fusing (in the correct reading frame) various restriction fragments of *GCR1* downstream from codon 202 of the *lexA* gene in plex1-202. Each fusion is indicated on the left of the figure and is named to reflect the GCR1 residues that are included [i.e. lexA−GCR1(12−844) contains all of the GCR1 protein between residues 12 and 844, inclusive]. Fusion gene *GCR1−lexA* was constructed by replacing *GCR1* codons 444−583 with codons 1−202 of *lexA*. Plasmids bearing these fusion genes were introduced into strain WG1 (Table II). In the column on the right, β-galactosidase enzyme activity is expressed as units/mg total protein. Stippled box, *in vitro* DNA binding domain (Huie *et al.*, 1992); filled box, *lexA* coding region. Restriction sites used to generate the fusion proteins are indicated as follows: 1, *Xmn*I; 2, *Sac*I; 3, *Eco*RV; 4, *Rsa*I; 5, *Nco*I; 6, *Pvu*I; 7, *Ava*I; 9, *Tth*111I.

domain of lexA and various regions of GCR1, and assayed the fusion proteins for the capacity to activate transcription of a reporter gene (*lacZ*) that contains two copies of the lexA operator in its promoter. All of the fusion genes except one (*GCR1−lexA*) fused regions of *GCR1* to the C-terminal end of *lexA*; in the *GCR1−lexA* fusion gene, the *lexA* coding region replaces *GCR1* codons 444−583. The results are summarized in Figure 5 and demonstrate that GCR1 contains an activation domain, and that this domain is located within the N-terminal third of the protein. Western blot analysis of the lexA−GCR1 fusion proteins was done by using a lexA antibody. The non-activating lexA−GCR1 fusion proteins were of the expected size (data not shown). Also, with the exception of the lexA−GCR1(12−844) protein, all of the lexA−GCR1 fusion proteins were present at equivalent levels [the lexA−GCR1 (68−844) protein and the GCR1−lexA fusion protein were not tested]. The weaker activation by the lexA−GCR1(12−844) fusion protein could be a result of its lower steady-state level relative to the smaller N-terminal fusions [lexA−GCR1(12−444), lexA−GCR1(12−367) and lexA−GCR1(68−844)] and the lexA−GAL4 control. Band retardation analysis with an oligonucleotide probe containing the lexA operator (also not shown) demonstrated that the fusion proteins retained the capacity to bind upstream from the *lacZ* reporter gene. These data suggest that the region of GCR1 between residues 68 and 367 contains an activation domain.

The region containing the activation domain is essential for GCR1 function

Our initial experiments indicated that removal of the C-terminal region containing the specific DNA binding domain (a.a. 690−844) resulted in at most a minor loss of GCR1 function. We next undertook a mutational analysis to investigate the *in vivo* requirement for the remainder of the

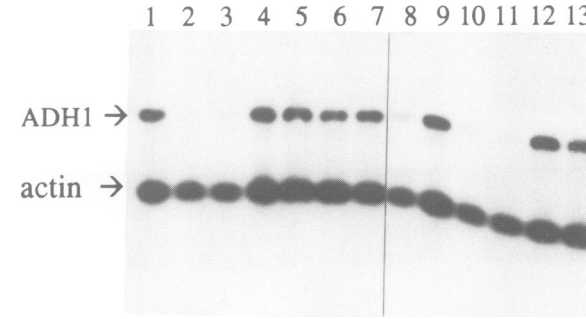

Fig. 6. Activation of transcription by *GCR1* genes with internal deletions. *ADH1* and actin mRNAs from *gcr1*⁻ cells (lane 8) or *gcr1*⁻ transformants (lanes 1−7 and 9−13) harvested during early logarithmic phase were subjected to S1 nuclease analysis. Equal amounts of total RNA were analyzed. S1-resistant products were separated in alkaline 2% agarose gels. Lanes: 1, wild type *GCR1*; 2, Δ11−211; 3, Δ212−367; 4, Δ368−443; 5, Δ444−583; 6, Δ584−651; 7, Δ368−651; 8, untransformed *gcr1*⁻ cells; 9, myc-tagged wild type *GCR1*; 10, myc-tagged Δ11−211; 11, myc-tagged Δ212−367; 12, myc-tagged Δ368−443; 13, myc-tagged Δ584−651.

GCR1 protein. We generated additional mutant GCR1 genes with in-phase deletions that encoded proteins missing various stretches of amino acids (Figure 1). The mutants were named to reflect the missing region; for example, mutant Δ11−211 is missing amino acids 11−211, inclusive, which have been replaced with the appropriate synthetic *Xba*I linker (to maintain the correct reading frame). The mutant genes Δ11−211 and Δ212−367 are lacking portions of the activation domain, while the mutant genes Δ368−443, Δ444−583, Δ584−651 and Δ368−651 are lacking part or all of the region between the activation domain and the specific DNA binding domain.

Transformants carrying the mutant genes with deletions in the N-terminal third of the *GCR1* coding region (mutants Δ11−211 and Δ212−367) were unable to complement the slow growth phenotype of *gcr1*⁻ cells (Figure 3e and f, respectively), producing colonies similar in size to untransformed gcr1⁻ cells (Figure 3b). In contrast, the mutant genes that contained deletions of the middle third (mutants Δ368−443, Δ444−583, Δ584−651 and Δ368−651) were able to complement the slow growth phenotype of gcr1⁻ cells (Figure 3g, h, i and j), generating colonies indistinguishable in size from those of wild type cells (Figure 3a). The internal deletion analyses thus tentatively identified the N-terminal region containing the activation domain as essential and the middle third of the protein as non-essential. The C-terminal truncation mutants demonstrated that the C-terminus of GCR1 was also not essential for GCR1 function.

To ensure that the defective mutated genes produced the expected products, we tagged each of the mutant GCR1 proteins with the myc epitope and assayed the level of each mutant protein by using Western blot analysis. The size of *gcr1*⁻ colonies transformed with the myc-tagged wild type (Figure 3k) or mutant genes (not shown) was the same as those transformed with the untagged counterparts. The results showed that the level of the mutant proteins that failed to complement the *gcr1*⁻ phenotype (Δ11−211 and Δ212−367) was equal to or greater than that generated by a wild-type gene present at low copy number (data not shown).

We also tested each of the internally deleted *GCR1* genes

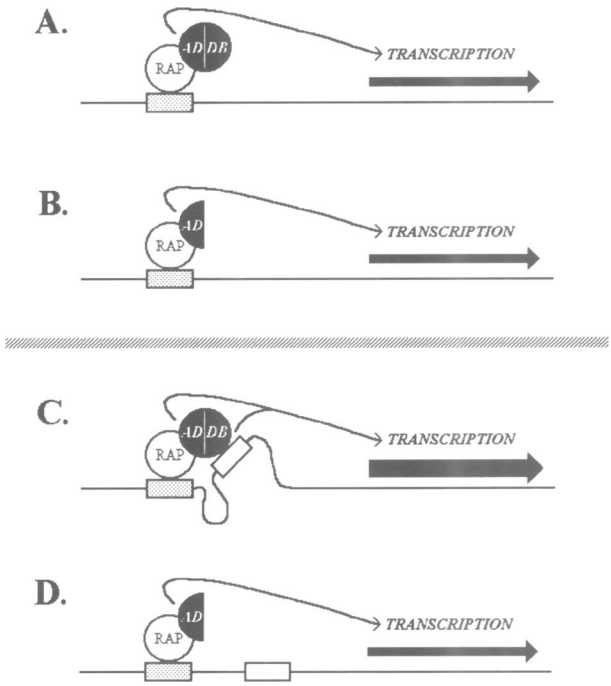

Fig. 7. A model for transcriptional activation by GCR1. **A.** A heteromeric complex that contains GCR1 (filled circle) and RAP1 (open circle) interacts with UAS_{RPG} (stippled rectangle) through the RAP1 DNA binding domain and contacts the transcriptional machinery through the GCR1 activation domain (AD). **B.** For promoters that contain UAS_{RPG} but lack a potentiator transcriptional activation by RAP1 and GCR1 is unaffected by removal of the GCR1 DNA binding domain (DB). **C.** For promoters that contain UAS_{RPG} and a potentiating CT box (open rectangle), both the activation domain and DNA binding domain of GCR1 function to activate high levels of transcription. The model predicts that the potentiator is involved but not necessarily sufficient to generate maximal levels of transcription. **D.** Removal of the GCR1 DNA binding domain curtails but does not eliminate GCR1-dependent transcriptional activation relative to panel C, which is indicated by the reduced width of the filled arrow. The filled arrows indicate similar levels of activation in panels A and B, but do not necessarily indicate that transcriptional levels in panels A and D are equivalent.

for its capacity to activate *ADH1* transcription. This experiment was designed to determine whether the capacity of each mutant GCR1 protein to correct the *gcr1*$^-$ growth defect was correlated with its capacity to activate transcription of *ADH1*, as was observed for the truncated GCR1. All the deletion mutants capable of complementing the *gcr1*$^-$ growth defect were also capable of activating *ADH1* transcription to wild type levels (Figure 6, untagged genes, lanes 4, 5, 6 and 7; myc-tagged genes, lanes 12 and 13). As expected, in neither of the growth impaired mutants (Figure 6, untagged genes, lanes 2 and 3; myc-tagged genes, lanes 10 and 11) did *ADH1* transcription exceed the level in *gcr1*$^-$ cells (Figure 6, lane 8).

These data suggest that the region between residues 1 and 368 contains the critical part of GCR1 and that the remainder of the protein (between residues 368 and 844) is not required for transcriptional activation of its target genes.

Discussion

GCR1 is required for high levels of transcription of the glycolytic genes and at least several translational component genes. The discovery of a specific DNA binding domain in the C-terminus of GCR1 led to the proposal of two hypotheses postulating that the binding of GCR1 to its specific site, the CT box, is sufficient to allow GCR1 access to its target promoters (Huie *et al.*, 1992; Uemura and Jigami, 1992; Scott and Baker, 1993). These hypotheses were inconsistent with two observations: the CT box does not act as an upstream activating sequence on its own (Bitter *et al.*, 1991) and several RAP1-dependent genes that lack a functional CT box are nevertheless GCR1-dependent (Santangelo and Tornow, 1990). In this paper, we report that the specific DNA binding domain of GCR1 is not required for GCR1-dependent transcriptional activation, a finding that also contradicts both hypotheses.

We have proposed an alternative hypothesis, namely that RAP1 and GCR1 are interdependent transcriptional activators (Santangelo and Tornow, 1990). This alternative is supported by several independent observations. An isolated RAP1 binding site, placed into a defined promoter that lacks CT boxes, activates transcription of the downstream reporter gene in a GCR1-dependent fashion (Santangelo and Tornow, 1990). Four other UAS_{RPG}-driven promoters lacking a functional CT box (*CRY1*, *TEF1*, *TEF2* and *RP29*) are likewise GCR1-dependent (Santangelo and Tornow, 1990; our unpublished data). Transcriptional activation by GCR1 depends more on UAS_{RPG} than the CT box (Table I) and GCR1 can be co-immunoprecipitated with RAP1 (Figure 4). Finally, we found that the $\Delta 444-844$ and $\Delta 535-844$ proteins activate transcription despite the removal of the entire specific DNA binding domain in the C-terminus (Figure 3). All of these data suggest that GCR1 functions while complexed with RAP1. There is genetic evidence to support the hypothesis that RAP1 and GCR1 interact: suppressor mutations that increase RAP1-mediated transcriptional activation of *HIS4* map in *GCR1* (Devlin *et al.*, 1991). In light of these data, the absence of functional CT boxes in translational component promoters does not weigh against the hypothesis that GCR1 and RAP1 activate transcription of those genes interdependently. It is therefore unnecessary to invoke an indirect effect of GCR1 on translational component gene expression, as was proposed by others (Huie *et al.*, 1992).

We report here that GCR1 contains an activation domain that lies within an essential region of the protein. The use of lexA−GCR1 fusion proteins allowed us to identify a region of GCR1 that is capable of activating transcription of a lexA-driven reporter gene when fused to the lexA DNA binding domain. A common criticism of this type of analysis is that segments of some proteins may function as activation domains artifactually when removed from the normal context of the entire protein. This was of particular concern to us because of the contrast between our results and those of Uemura and Jigama (1992), in which a GAL4−GCR1 fusion protein did not activate transcription. To address this issue, we also asked whether the entire GCR1 protein could activate transcription when fused to the lexA DNA binding domain, and found that it could (Figure 5). The negative result obtained by Uemura and Jigami could be explained by either of two possibilities that they did not test—low levels of their GAL4−GCR1 fusion protein or its failure to bind UAS_G. We therefore believe that GCR1 is capable of activating transcription and that the region between residues 68 and 367 contains the domain required for this activity. Visual inspection of this region revealed the presence of a potential amphipathic α-helix. Within the putative amphipathic α-helix

Table II. Strains and plasmids

Strain or plasmid	Characteristics or genotype	Source or reference
Strains		
S173-6B(GCR1$^+$)	*Mat-α leu2−3,112 his3-1 ura3-52 trp1-289*	Holland *et al.* (1987)
gcr1$^-$	*MATα leu2-3, 112 his3-1 ura3-52 trp1-289 gcr1*Δ1::URA3	Holland *et al.* (1987)
WG1	same as S173-6B and *ura3::pHH199 (URA3)*	This work
CY1658	*MATα ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11,15 ura 3-1* Δ*rap1::LEU2* Δ*gcr1::HIS3* with YCp50-RAP1 and YEpTRP-GCR1(myc$_2$)	Kim Arndt
CY1662	*Matα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1* Δ*rap1::LEU2* Δ*gcr1::HIS3* with YCp50-RAP1(HA$_2$) and YEpTRP-GCR1(myc$_2$)	Kim Arndt
Plasmids		
YEp13	2μ ori *LEU2* Amp^R pMB1 ori	Broach *et al.* (1979)
YEpTRP	2μ ori *TRP1* Amp^R pMB1 ori	This work
plex1-202	2μ ori *HIS3* Amp^R pMB1 ori *lexA* fusion gene driven by *ADH1* promoter with polylinker at *lexA* a.a. 202	Himmelfarb *et al.* (1990)
pHH199	*URA3* Amp^R pMB1 ori *lacZ* reporter gene with two lexA operator sites in the promoter	Himmelfarb *et al.* (1990)
pPC0	2μ ori *LEU2* Amp^R pMB1 ori promoterless ADH1 and *LAC4* reporter genes divergently arrayed	Santangelo *et al.* (1988)
pPC0(−615)	pPC0 with −3 to −615 of the *ADH1* promoter fused to *LAC4*	This work

(located between amino acids 264 and 300) is a 5/5 match to GCN4 and a 5/6 match to VP16 with respect to the placement of bulky hydrophobic residues found to be important for activation *in vivo* (Cress and Triezenberg, 1991). We are currently trying both to find out whether or not the putative amphipathic α-helix is a requisite part of the activation domain and to explain the apparent importance of the remainder of the essential N-terminal region.

GCR1−RAP1 interdependence therefore appears to be manifested through a GCR1−RAP1 heteromeric complex that interacts with UAS$_{RPG}$ through the RAP1 DNA binding domain and contacts the transcriptional machinery through the GCR1 activation domain (Figure 7A). We have not ruled out the possibility that GCR1−RAP1 heteromers also bind to the CT box if one is present, as appears to occur in the *TP1* promoter (Huie *et al.*, 1992), but the functional significance of CT box recognition by GCR1 remains unclear. For promoters with a 'potentiating' CT box (Buchman *et al.*, 1988; Bitter *et al.*, 1991), the interaction between GCR1−RAP1 complexes and the promoter DNA may be stabilized so that transcriptional activation is more efficient (Figure 7C). This effect could simply result from better retention of GCR1 by promoter DNA when two surfaces (RAP1 and the CT box) interact independently with it. Alternatively, binding to the CT box may just be an effective means of getting the C-terminus of GCR1 out of the way of the activation domain. If so, the presence of CT boxes in RAP1-dependent promoters, a common occurrence in glycolytic genes, would increase the efficiency of an intact GCR1 activator, a scenario that might also have regulatory consequences. Removal of the C-terminus of GCR1 (as in the Δ535−844 protein) would result in a constitutive activator that is CT box-independent.

A model predicting that a GCR1−RAP1 complex activates transcription explains other observations about RAP1, which is a highly abundant protein with multiple functions in the cell, including repression of transcription (Shore and Nasmyth, 1987; Shore *et al.*, 1987) and telomere maintenance (Runge and Zakian, 1989; Lustig *et al.*, 1990). RAP1 might accomplish different tasks by forming complexes with different proteins that provide the domains specific to each task. In support of this idea, a protein that is involved in transcriptional silencing and telomere maintenance was identified by virtue of its interaction with RAP1 (Hardy *et al.*, 1992). According to our model, when RAP1 is involved in transcriptional activation it complexes with GCR1, which provides the heteromer with an activation domain.

In conclusion, our results suggest an unusual epistatic relationship between a transcriptional activator (GCR1) with its own DNA binding and activation domains, and a second DNA binding protein (RAP1) on which it appears to depend for access to target promoters. Subsequent analysis of this relationship may suggest how two eukaryotic transcription factors co-operate to regulate gene expression.

Materials and methods

Strains, plasmids and media

The strains and plasmids used in this study are described in Table II. Yeast cells were grown in minimal medium (yeast nitrogen base; Difco), supplemented with the appropriate amino acids (40 mg/l) and either 2% glucose (YNBD) or 3% pyruvate (YNBpyr).

Yeast transformations

Yeast cells were transformed by the lithium acetate method of Ito *et al.* (1983), with the modification of adding 80 μg of denatured sheared salmon sperm DNA before the addition of the plasmid DNA. Transformants were selected on YNBD agar containing 40 mg/l of all necessary amino acids except either leucine (for YEp13 derivatives), tryptophan (for YEpTRP derivatives), or histidine (for plex1-202 derivatives).

β-galactosidase assays

Yeast transformants were grown in YNBpyr [for the pPC0(−615)UAS$_{RPG}$ and pPC0(−615)UAS$_{RPG}$* experiments] or YNBD (for the *lexA−GCR1* fusion gene experiments) to mid-logarithmic phase. The cells were harvested by centrifugation and broken by adding glass beads and agitating the mixture vigorously with a vortex mixer. β-galactosidase was assayed as described previously by Santangelo *et al.* (1988). Protein concentrations were determined by using the BCA Assay (Pierce, Rockville, IL).

Epitope tagging of GCR1

A 39mer oligonucleotide with the following sequence was synthesized on a Milligen DNA synthesizer: (5′-CTGGAGCAAAAGCTGATTTCT-GAGGAGGATCTCGGATCCG-3′). This oligonucleotide contains the coding information for a 10 amino acid c-myc epitope (bases 3−32) recognized by the monoclonal antibody 9E10 (Evan *et al.*, 1985). A single

copy was inserted at the unique *Pvu*II site in the wild type and mutant *GCR1* genes cloned in the YEpTRP vector (except Δ444−583 and Δ368−651, in which the *Pvu*II site has been deleted). To increase the sensitivity of detection of GCR1 in the co-immunoprecipitation experiments, a second copy of the oligonucleotide was inserted into the wild type gene at the same location.

Cell labeling and immunoprecipitation

Labeled extracts were prepared by growing yeast cells in YNBD. The cells were harvested, resuspended in YNBD containing 50 μCi/ml of ^{35}S labeling mix (Dupont NEN, Boston, MA) for 1 hour. The radiolabeled cells were then harvested, resuspended to 200 OD_{600} units/ml in breaking buffer (100 mM Tris pH 7.0, 200 mM NaCl, 1 mM EDTA, 5% glycerol and 0.5 mM DTT) and then lysed with glass beads. Unlabeled extracts were prepared identically by omitting the labeling step.

Immunoprecipitations were done by incubating each extract with anti-HA antibody in the presence of PMSF (1 mM) and protease inhibitors (aprotinin, leupeptin, chymostatin and pepstatin; each at final concentration of 0.8 μg/ml) for 1 hour on ice. The reaction was spun at 13 000 *g* for 15 min at 4°C and the supernatant was transferred to a new tube containing protein G−Sepharose beads in 0.3 × RIPA buffer (1 × = 50 mM Tris pH 7.0, 1% Triton ×100, 0.5% sodium deoxycholate, 0.1% SDS and 200 mM NaCl) that had been preincubated in fetal calf serum. The reaction was incubated at 4°C for 1 hour with rocking. The beads were pelleted by low speed centrifugation, washed twice with wash buffer (75% breaking buffer and 25% 0.3 × RIPA buffer, plus 1 mM PMSF) and once with final wash buffer (50 mM Tris pH 7.0 and 50 mM NACl). After the last spin, care was taken to completely remove all the final wash buffer. The proteins bound to the protein G−Sepharose beads were eluted by adding gel loading buffer (Laemmli, 1970) and heating to 100°C for 10 min. The samples were separated by electrophoresis in a 7.5% polyacrylamide−0.1% SDS gel (Laemmli, 1970). After electrophoresis, radioactive gels were fixed (30% methanol and 10% acetic acid), treated with Entensify (Dupont NEN, Boston, MA), then dried and exposed to Kodak XAR5 film with an intensifying screen. The proteins in unlabeled gels were transferred to nitrocellulose and the probed with the anti-myc antibody according to the standard Western blot method (Burnette, 1981). Antibody−antigen complexes were detected with the ECL detection reagents (Amersham, Arlington Heights, IL).

Band retardation assays

Protein extracts were prepared as described previously by Santangelo and Tornow (1990). 1 ng of radiolabeled UAS_{RPG} oligonucleotide (Santangelo and Tornow, 1990) was incubated with whole cell extract of CY1662 cells in binding buffer (Santangelo and Tornow, 1990). For the ethidium bromide interference experiment, the oligonucleotide and protein extract were preincubated, to allow RAP1−DNA complexes to form, prior to the addition of the ethidium bromide. Protein−DNA complexes were resolved in 3% Nusieve agarose, 0.5 × TBE buffer, as described previously by Santangelo and Tornow (1990).

Deletion mutagenesis

All internal deletion mutants were constructed by following the same basic scheme: plasmids containing *GCR1* were digested in parallel with two different restriction enzymes. The appropriate fragments from each digest were then isolated and ligated together to generate a plasmid containing a *GCR1* gene in which the sequence information between the two restriction sites has been replaced with a single *Xba*I site. DNA sequence analysis of each deletion junction confirmed that the desired deletion had been made and that the reading frame was unchanged. An 8.3 kb *Bgl*II−*Xho*I fragment [containing the entire wild type (or mutant) *GCR1* gene as well as 5′ and 3′ flanking sequences] was cloned into either YEp13 or YEpTRP.

RNA isolation and analysis

Total RNA was extracted from cells grown in YNBD and analyzed as described previously by Santangelo *et al.* (1988). RNA was hybridized to an excess of actin-specific and either *ADH1*- or *TEF*-specific radiolabeled DNA probes and then digested with S1 nuclease, as described previously by Santangelo and Tornow (1990) and Tornow and Santangelo (1990).

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